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ORIGINAL ARTICLE

Characterisation of *mecA* gene negative *Staphylococcus aureus* isolated from bovine mastitis milk from Northern Germany

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Abstract

Staphylococcus aureus (*S. aureus*) is an important causative agent of contagious intermammary infections in dairy cattle. *S. aureus* is also considered as an important foodborne pathogen and cause of food poisoning cases and outbreaks worldwide. In order to understand the molecular ecology of *S. aureus*, the present study compared phenotypic and genotypic characteristics of 70 *S. aureus* isolates from bovine mastitis milk samples collected during the period from August 2001 to March 2014 in different regions of Northern Germany. The *S. aureus* isolates were characterised phenotypically, as well as genotypically for their genetic diversity using multi-locus sequence typing (MLST), *spa* typing and the presence of virulence genes encoding 16 staphylococcal enterotoxins (*sea-selu*), toxic shock syndrome toxin (*tst*), thermonuclease (*nuc*), clumping factor (*clfA* and *clfB*), coagulase (*coa*) and the methicillin resistance gene *mecA*. A total of 16 sequence types were grouped into eight clonal complexes (CCs), and 17 *spa* types were identified. These included six novel sequence types and one novel *spa* type. The majority of bovine mastitis milk-associated sequence types belonged to the clonal complex CC5, CC97, CC133, and CC151 and showed closely related genotypes or lineages with sequence types of human origin. The genotype CC133 (ST133-t1403) was predominant, constituting 27.1% of the isolates. In addition, the *S. aureus* isolates displayed nine different enterotoxigenic profiles. All *S. aureus* were methicillin-susceptible (MSSA). The current study provides new information on phenotypic and genotypic traits of *S. aureus* isolates from bovine mastitis. The comparison of characteristics of isolates from the present study originating from mastitis milk showed similarities with human isolates. This might help to better understand the distribution of *S. aureus* in the one health context.

Introduction

Staphylococcus aureus (*S. aureus*) is one of the most important etiological agents of contagious clinical and subclinical mastitis in dairy herds and other mammals worldwide (Peton and Le Loir 2014). The bacterium causes significant economic losses in the dairy industry by affecting both the quality and quantity of milk produced, premature slaughter and veterinary and treatment costs (Hogeveen et al. 2011). *S. aureus* is an ubiquitous pathogen, found on many biotic and abiotic components connected with dairy production, e.g. mammary gland, teat, feed stuff, milking personnel, insects, non-bovine animals, as well as on milking and farm equipment (Anderson et al. 2012; Kerro Dego et al. 2002). Besides the mere detection and identification of *S. aureus* isolated from bovine mastitis, it is important to know the characteristics of the isolates in order to gain a more detailed insight into the epidemiological distribution of different genotypes as well as to identify the dispersion of genotypes having an impact on human and animal health (Fitzgerald and Holden 2016). In a recent report, the European Food Safety Authority (EFSA) recommends monitoring foodborne animals for methicillin-resistant *S. aureus* (MRSA) and systematic surveillance in humans to better identify trends in the dissemination or evolution of these bacteria (EFSA ECDC 2017) Despite *S. aureus* harbouring antimicrobial resistance, food may become

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contaminated with enterotoxin-producing *S. aureus* (Argudin et al. 2010). In addition to enterotoxins, various virulence factors of *S. aureus* are known to cause infection. The virulence factors are divided into several categories, e.g., surface-associated factors, degradative enzymes and superantigen toxins. Staphylococcal superantigens (SAg) include staphylococcal enterotoxins (SEs), staphylococcal enterotoxin-like proteins (SEls) and toxic shock syndrome toxin-1 (TSST-1; Xu and McCormick 2012). The SEs in particular are involved in human food poisoning (Hennekinne et al. 2010).

The aim of the present study was to contribute to the epidemiological knowledge of *S. aureus* related to bovine mastitis cases.

Isolates were characterised by determining phenotypic and genotypic properties in correlation with genes encoding toxins, together with analysing the *mecA* gene to identify the presence of MRSA. In addition, the public health aspect was addressed by relating the obtained isolates to isolates of human origin already published in MLST databases. This adds to the knowledge regarding the risk for personnel involved in dairy production and especially to the exposure of consumers to raw dairy products.

Methods

Isolation, identification and phenotypic characterisation of the *S. aureus* isolates

In the present study, $n = 70$ *S. aureus* isolates originating from bovine mastitis milk collected from August 2001 to March 2014 on dairy farms of Northern Germany were characterised. Following German (DVG 2009) and international standards (NMC 2017), udder health was categorised by means of somatic cell count (threshold: 100.000/ml quarter foremilk) and presence of pathogenic bacteria (in the case of *S. aureus*: 3 cfu/ml). In these standards which are used for laboratory work in which the affected animals cannot be inspected personally by the sample-processing staff, the combination of an elevated somatic cell count and a positive microbiological finding is termed 'mastitis' (as opposed to, e.g. 'unspecific mastitis' which refers to high cell counts plus a negative bacteriological result or 'latent infection' with a positive bacteriological finding but cell counts below the threshold), regardless its clinical manifestation. In this way, strains are derived from both clinical and subclinical cases sent in by veterinary practitioners from several herds in Northern Germany. Udder health assessment is a routine service offered by the Institute of Food Quality and Food Safety, University of Veterinary Medicine, Foundation, Hannover, Germany. In all cases, strains originated from quarter foremilk samples. If practitioners handed in other milk fraction samples, isolated strains were excluded from the present analysis, as were repeated measurements (i.e. several *S. aureus* strains from one and the same cow).

The isolation and confirmation of *S. aureus* from milk samples were conducted using the method proposed by the German Veterinary Association's recommendations (DVG 2009). In short, samples were grown on aesculin blood agar plates (Oxoid, Wesel, Germany). Presumptive *S. aureus* were identified on the basis of morphological colony features (shiny, yellow convex colonies), β-haemolysis and positive catalase test. Staphaurex latex agglutination test (clumping factor and protein A; Oxoid, Altrincham, England) and additional coagulase testing using rabbit plasma (Becton, Dickinson, Heidelberg, Germany) for latex agglutination-negative isolates were employed. *S. aureus* presumptive isolates were finally identified by biochemically testing the isolates with the ID32-Staph identification system (bio-Mérieux Deutschland GmbH, Nürtingen, Germany). Tests were read visually, and the test results were evaluated by the apiweb (bio-Mérieux) online application for interpretation of results.

Testing for the presence of staphylococcal virulence factors and *mecA* gene

The *S. aureus* isolates were tested for various virulence genes and the *mecA* gene for identifying MRSA. The virulence gene typing included the following: thermonuclease (*nuc*); clumping factor (*clfA*); clumping factor (*clfB*); coagulase (*coa*) and staphylococcal enterotoxins SEA (*sea*), SEB (*seb*), SEC (*sec*), SEE (*see*), SEG (*seg*), SEH (*seh*), SEI (*sei*), SElJ (*selj*), SEM (*sem*), SEN (*sen*), SEO (*seo*), SEP (*sep*), SEQ (*seq*), SER (*ser*), SElU (*selu*) and TSST (*tst*). The applied primers, PCR conditions and references for the protocols are shown in Table 1. Chromosomal DNA of *S. aureus* isolates was extracted by means of the DNeasy Blood and Tissue kit in accordance with the manufacturer's protocol (Qiagen, Hilden, Germany). The total volume of polymerase chain reaction (PCR) mixture was 30 μL, consisting of 1 μL of each forward and reverse primer (each 10 pmol/μL; Eurofins Genomics, Ebersberg, Germany), 15 μL of 2 × Red Y Gold Mix Master containing (1 unit GoldStar DNA polymerase, 200 μM dNTPs, 1.5 μM $MgCl_2$, 20 μM $(NH_4)_2SO_4$, 75 μM Tris-HCl (pH 8.8 at 25 °C), 0.01% (*v*/*v*) Tween 20™ and Red Dye Loading Buffer; Eurogentec Deutschland, Cologne, Germany) and 10.5 μL of nuclease-free water (Qiagen). Finally, 2.5 μL DNA template of *S. aureus* was added to each reaction tube. The PCR products were determined by gel electrophoresis in 2% agarose gel (Peqlab, Erlangen, Germany). *S. aureus* strains producing SEA (619/93), SEB (62/92), SEC (1229/93), SED (1644/93), SEE (FRI 918), TSST-1 (161/93), SEG/SEI (Ly 990055) and SEH (Ly 990552), SEJ (2724)

Table 1 Primers and PCR conditions for amplifying virulence and enterotoxin genes of *S. aureus*

Gene	Primer	Sequence	Amplicon size (bp)	PCR conditions	Reference
clfA	clfA-1 clfA-2	5-ATTGGCGTGGCTTCAGTGCT-3 5-CGTTTCTTCCGTAGTTGCATTTG-3	288	1	(Tristan et al. 2003)
clfB	clfB-1 clfB-2	5-ACATCAGTAATAGTAGGGGCAAC-3 5-TTCGCACTGTTTGTGTTTGCAC-3	203	1	(Tristan et al. 2003)
coa	coa-1 coa-2	5-ATAGAGATGCTGGTACAGG-3 5-GCTTCCGATTGTTCGATGC-3	674–917	1	(Quinn et al. 2011)
mecA	MEC A-1 MEC A-2	5-GTGAAGATATACCAAGTGATT-3 5-ATGCGCTATAGATTGAAAGGAT-3	147	1	(Zhang et al. 2005)
nuc	nuc-1 nuc-2	5-CCTGAAGCAAGTGCATTTACGA-3 5-CTTTAGCCAAGCCTTGACGAACT-3	166	1	(Graber et al. 2007)
sea	SEA-1 SEA-2	5-AAAGTCCCGATCAATTTATGGCTA-3 5-GTAATTAACCGAAGGTTCTGTAGA-3	219	1	(Tsen and Chen 1992)
seb	SEB-1 SEB-2	5-TCGCATCAAACTGACAAACG-3 5-GCAGGTACTCTATAAGTGCC-3	478	1	(Johnson et al. 1991)
sec	SEC-1 SEC-2	5-GACATAAAAGCTAGGAATTT-3 5-AAATCGGATTAACATTATCC-3	257	1	(Johnson et al. 1991)
sed	SED-1 SED-2	5-CTAGTTTGGTAATATCTCCT-3 5-TAATGCTATATCTTATAGGG-3	317	2	(Johnson et al. 1991)
see	SEE-1 SEE-2	5-TACCAATTAACTTGTGGATAGAC-3 5-CTCTTTGCACCTTACCGC-3	171	1	(Pereira et al. 2009)
seg	SEG-1 SEG-2	5-AATTATGTGAATGCTCAACCCGATC-3 5AAACTTATATGGAACAAAAGGTACTAGTTC-3	642	1	(Jarraud et al. 1999)
seh	SEH-1 SEH-2	5-CAATCACATCATATGCGAAAGCAG-3 5-CATCTACCCAAACATTAGCACC-3	375	1	(Jarraud et al. 1999)
sei	SEI-1 SEI-2	5-CTCAAGGTGATATTGGTGTAGG-3 5-AAAAAACTTACAGGCAGTCCATCTC-3	576	1	(Jarraud et al. 1999)
selj	SElJ-1 SElJ-2	5-CATCAGAACTGTTGTTCCGCTAG-3 5-CTGAATTTTACCATCAAAGGTAC-3	142	1	(Monday and Bohach 1999)
sem	SEM-1 SEM-2	5-TCTTAGGAACTATTATGGTAGC-3 5-CCTGCATTAAATCCAGAA-3	471	1	(Akineden et al. 2008)
sen	SEN-1 SEN-2	5-GGAGTTACGATACATGATGG-3 5-ACTCTGCTCCCACTGAAC-3	292	1	(Akineden et al. 2008)
seo	SEO-1 SEO-2	5TGATGATTATATAAATAATCGATTTACG-3 5-ATATGTACAGGCAGTATCC-3	249	2	(Akineden et al. 2008)
sep	SEP-1 SEP-2	5-ATCATAACCAACCGAATCAC-3 5-AGAAGTAACTGTTCAGGAGCTA-3	148	1	(Chiang et al. 2008)
seq	SEQ-1 SEQ-2	5-TCAGGTCTTTGTAATACAAAA-3 5-TCTGCTTGACCAGTTCCGGT-3	359	1	(Chiang et al. 2008)
ser	SER-1 SER-2	5-AGATGTGTTTGGAATACCCTAT-3 5-CTATCAGCTGTGGAGTGCAT-3	123	1	(Chiang et al. 2008)
selu	SElU-1 SElU-2	5-ATTTGCTTTTATCTTCAT-3 5-GGACTTTAATGTTTGTTTCTGAT-3	167	3	(Chiang et al. 2008)
tst	TSST-1 TSST-2	5-GCTTGCGACAACTGCTACAG-3 5-TGGATCCGTCATTCATTGTTAT-3	559	1	(Lovseth et al. 2004)

PCR conditions: 135 cycles (94 °C, 30 s; 55 °C, 30 s; 72 °C; 30 s); 2: 35 cycles (94 °C, 30 s; 53 °C, 30 s; 72 °C, 30 s); 3: 35 cycles (94 °C, 30 s; 51 °C, 30 s; 72 °C, 30 s)

were collected from the strain collection of Dairy Sciences, Institute of Veterinary Food Science, Justus-Liebig-University Giessen, Germany, and used as a positive control in the PCR analysis (Taban et al. 2017). Likewise, the *S. aureus* strain 120/14 also served as positive control to determine the *mecA* gene. This was obtained from the Institute of Animal Hygiene, Animal Welfare and Ethology, University of Veterinary Medicine Hannover, Foundation, Germany. *S. aureus* ATCC 6538 was used as a positive control for the *nuc* gene.

Multi-locus sequence typing

Multi-locus sequence typing (MLST) was carried out with primers that had been previously designed by Enright et al. (2000) for detecting seven *S. aureus* housekeeping genes (Table 2). Amplicons were sequenced by Seqlab (Hann-vogt, Göttingen, Germany). DNA sequences were assembled by using the BioNumerics software v7.5 (Applied Maths, Sint-Martens-Latem, Belgium). The sequence types (ST) of the 70 study isolates were compared to the profiles of 33 *S. aureus* isolates from bovine mastitis from different European countries and 118 human *S. aureus* isolates from Germany, that were obtained from the MLST database (http://saureus.mlst.net), being the only isolates available at that moment of this part of the investigation. The relationship among the isolates was defined via the seven housekeeping genes identifying the ST. Clustering of MLST profiles was completed using a categorical coefficient and the isolates were clustered in groups. Sequence types according to MLST analysis were randomly formed into groups of closer relationships when at least five out of seven allele loci of the housekeeping genes were identical. Furthermore, minimum spanning trees were created in BioNumerics v7.5.

Protein A (*spa*) typing

The *spa* typing was performed with specific primers which were previously described by Shopsin et al. (1999; Table 2). Staphylococcal protein A contains a specific repeat region that was amplified and afterwards sequenced. All the *spa* repeats and typing were assigned by using the BioNumerics software v7.5. Numeric *spa* typing and *spa* type codes were determined in accordance with the Ridom *Spa* Server website (www.spaserver.ridom.de).

Results and discussion

Microbiological identification and phenotypic characterisation of the *S. aureus* isolates

In the present study, phenotypic and genotypic methods were used to characterise 70 *S. aureus* isolates from bovine mastitis milk samples. Among these, 27 isolates (38.6%) showed α-haemolysis, 26 (37.1%) β-haemolysis and 17 (24.2%) were non-haemolytic. The Staphaurex (clumping factor and protein A) test was positive in 75.7% of the isolates ($n = 53$). The ID32-Staph identification system also confirmed all 70 isolates to be *S. aureus*. The rate of identification reliability of 64 isolates was ID 95% to 99.8%, based on the ID32s identification reliability scores (ID) read from the apiweb application. For the remaining six isolates, the ID percentage was lower and as low as 62% in the case of three isolates. According to the results from the biochemical reactions and also based on the presence of the *nuc* and *coa* genes, all isolates were correctly identified as *S. aureus*. Regarding correctly identifying *S. aureus* in mastitis cases, it should be pointed out not to rely on screening tests like latex agglutination alone as it was shown that a good proportion of isolates could be missed because of negative test results while still being case-relevant (Kamaleldin et al. 2010; Stutz et al. 2011). The study isolates were also all classified as methicillin-susceptible *S. aureus* (MSSA) on the basis of the absence of the *mecA*

Table 2 Primers and PCR conditions used in the MLST and *spa*-typing

Gene	Primer	Sequence (5–3)	Amplicon size (bp)	Reference
Carbamate kinase (*arcC*)	arcC-1 arcC-2	5-TTGATTCACCAGCGCGTATTGTC-3 5-AGGTATCTGCTTCAATCAGCG-3	569	(Enright et al. 2000)
Shikimate dehydro-genase (*aroE*)	aroE-1 aroE-2	5-ATCGGAAATCCTATTTCACATTC-3 5-GGTGTTGTATTAATAACGATATC-3	535	(Enright et al. 2000)
Glycerol kinase (*glpF*)	glpF-1 glpF-2	5-CTAGGAACTGCAATCTTAATCC-3 5-TGGTAAAATCGCATGTCCAATTC-3	575	(Enright et al. 2000)
Guanylate kinase (*gmk*)	gmk-1 gmk-2	5-ATCGTTTTATCGGGACCATC-3 5-TCATTAACTACAACGTAATCGTA-3	487	(Enright et al. 2000)
Phosphate acetyltrans-ferase (*pta*)	pta-1 pta-2	5-GTTAAAATCGTATTACCTGAAGG-3 5-GACCCTTTTGTTGAAAAGCTTAA-3	574	(Enright et al. 2000)
Triosephosphate isomerase (*tpi*)	tpi-1 tpi-2	5-TCGTTCATTCTGAACGTCGTGAA-3 5-TTTGCACCTTCTAACAATTGTAC-3	470	(Enright et al. 2000)
Acetylcoenzyme A ace-tyltransferase (*yqil*)	yqiL-1 yqiL-2	5-CAGCATACAGGACACCTATTGGC-3 5-CGTTGAGGAATCGATACTGGAAC-3	597	(Enright et al. 2000)
spa	SPA-1 SPA-2	5-AGACGATCCTTCGGTGAGC-3 5-GCTTTTGCAATGTCATTTACTG-3	variable	(Shopsin et al. 1999)

PCR conditions: 35 cycles (94 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s)

gene. A high proportion of MSSA in milk and dairy product samples has been reported, although MRSA can be present as well (Haran et al. 2012).

Multi-locus sequence typing

In the present study, the 70 isolates comprised 16 different sequence types (ST, Table 3), of which six were novel STs designated as ST2821 ($n = 2$), ST2823 ($n = 1$), ST2824 ($n = 1$), ST2825 ($n = 2$), ST2826 ($n = 1$) and ST2827 ($n = 1$). These new STs were submitted as new registrations to the MLST database at http://saureus.mlst.net. The novel STs indicated an evolutionary emergence of unique clones in the different study regions and their importance remains to be evaluated. Despite the novel types, the most common sequence types were ST133 ($n = 20$), ST504 ($n = 16$) and ST97 ($n = 11$). The number of other STs each was below five for ST398, ST479, ST1380, ST151, ST7, ST71 and ST464. As shown in Table 3, STs belonged to eight clonal complexes (CCs). Of these, CC133, represented by ST133 and ST2821, was the most prevalent genotype (31.4%, 22/70), followed by CC151 (27.1%, 19/70) and CC97 (21.4%, 15/70). The latter was the most diverse clonal complex, consisting of five different ST. The other clonal complexes were less prevalent. Research has shown that the majority of ruminant-associated STs belong to CC133, CC151 and CC97 (Guinane et al. 2010). However, these CC are known not to be species-specific. Regarding the most frequently encountered CCs, CC133/ST133 was isolated from other animals like small ruminants (Guinane et al. 2010), ungulates, rodents, felids (Espinosa-Gongora et al. 2012; Sasaki et al. 2012), or wild boars (Meemken et al. 2013), CC97 from pigs (Battisti et al. 2010). Four isolates belonged to CC398 which recently received a lot of attention because of containing the livestock-associated MRSA (Huijsdens et al. 2006; Lewis et al. 2008; Nemati et al. 2008). Our study revealed that all CC398 isolates were MSSA.

In order to describe the relationship between the *S. aureus* bovine mastitis isolates of this study and those from different European countries, a minimum spanning tree was set up, merging STs of the study isolates with STs from the 33 European bovine mastitis isolates taken from the MLST database. In total, seven groups were defined (A–G) based on a cut-off of more than two differing sequences of the seven alleles of the MLST analysis. Group A (CC133) was related to a French isolate. In group B (CC5), ST2825 was related to

Table 3 The genotypes of *S. aureus* isolates determined by the molecular typing methods and the relationship between sequence types (ST) of MLST and protein A (*spa* type)

Clonal complex	MLST type	*spa* type	No. of isolates	%	ID
CC133	ST133	t1403	19	27.1	5397, 5398
	ST133	t528	1	1.4	
	ST2821*	t1403	2	2.9	
CC151	ST504	t529	16	22.9	5401
	ST151	t529	2	2.9	
	ST2823*	t529	1	1.4	
CC97	ST97	t521	7	10	5402
	ST97	t13769*	1	1.4	
	ST97	t359	1	1.4	
	ST97	t267	1	1.4	
	ST97	t5920	1	1.4	
	ST71	t524	1	1.4	
	ST464	t3297	1	1.4	
	ST2824*	t5180	1	1.4	
	ST2826*	t521	1	1.4	5404
CC479	ST479	t528	2	2.9	
	ST479	t2873	1	1.4	
	ST1380	t2873	2	2.9	
	ST1380	t528	1	1.4	
CC398	ST398	t34	3	4.3	
	ST398	t571	1	1.4	
CC8	ST7	t91	1	1.4	
CC5	ST2825*	t586	2	2.9	5403, 5406
CC50	ST2827*	t519	1	1.4	5405

*Novel sequence types and *spa* type found in the present study; ID: reference identification number for new STs in accordance with the MLST database at http://saureus.mlst.net

isolates from England, the Netherlands and Germany, while in group C (CC8), ST7 was related to another German isolate (Fig. 1). In group D, two isolates from England clustered with an isolate from the Netherlands. Groups E–G included only isolates from the present study. Besides comparison with European database isolates of bovine mastitis cases, ST133/CC133 was found in other animals as mentioned above. ST504, on the other hand, seems to have been exclusive to bovine mastitis until now (http://www.mlst.net). ST97 was associated with bovine mastitis in the Netherlands (Kozytska et al. 2010) and Denmark (Hasman et al. 2010), but has also been reported in humans in Spain and the UK (Lozano et al. 2011; Sung et al. 2008).

Spa types

Isolates were grouped into 17 *spa* types (Table 3). One new *spa* type (t13769) was found. The most frequent *spa* types were t1403 ($n = 21$), t529 ($n = 19$) and t521 ($n = 8$), with the rest occurring fewer than five times. Several studies showed the similarity among *spa* types of *S. aureus* from different kinds of food, animals and humans. A high percentage of t1403 was identified in bovine mastitis isolates from Germany (Johler et al. 2011) and Sweden (Smyth et al. 2009). Some recent studies from Germany, Japan and Switzerland reported the *spa* type t529 in *S. aureus* isolated from bovine milk (Hata et al. 2010; Monecke et al. 2007) or dairy cattle (Veh et al. 2015). This shows the complexity of the epidemiological situation for the different varieties of *S. aureus* in general. The combination of ST and *spa* types proved to be useful, as *spa* typing has been used to identify most common ancestor lineages. Our results were in agreement with previous studies which detected ST151-t529 in *S. aureus* isolated from bovine milk (Hasman et al. 2010; Johler et al. 2011). Nineteen of the ST133 isolates belonged to *spa* type t1403, one to type t528. ST133-t1403 was found in bovine mastitis milk in Sweden, Germany and Denmark (Hasman et al. 2010; Johler et al. 2011; Smyth et al. 2009). Likewise, 16 of the ST504 isolates belonged to *spa* type t529 which was already known from Swiss bovine mastitis isolates (http://saureus.mlst.net/). All ST97 isolates were split into 11 *spa* types (Table 3). The results of this study were in agreement with other studies which detected ST97-t521, ST97-t267 and ST97-t359 in *S. aureus* isolated from cows and bovine mastitis (Hasman et al. 2010; Hata et al. 2010) as well as in humans in Brazil (www.spaserver.ridom.dewww.spaserver.ridom.de).

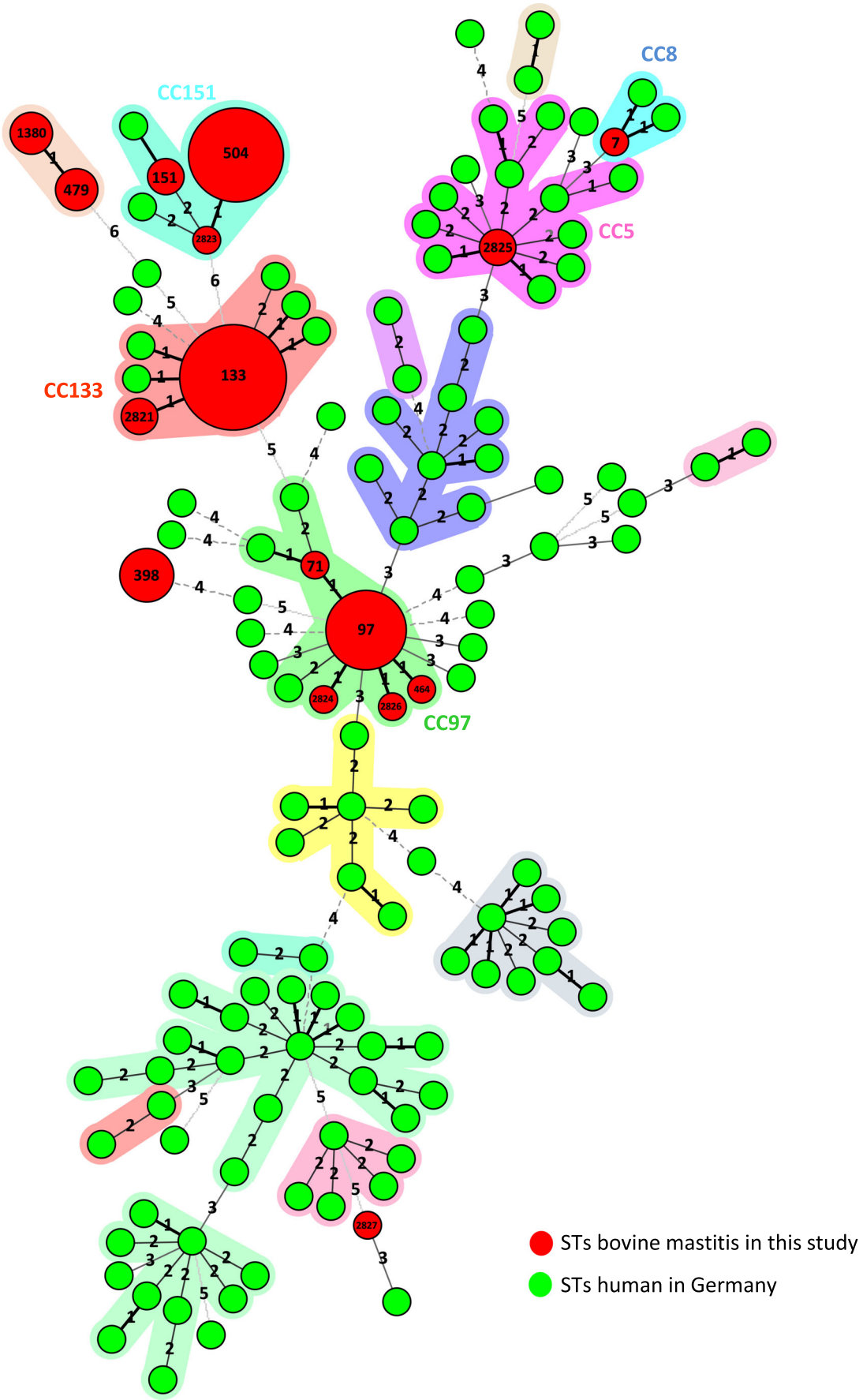
Relationship between isolates from bovine mastitis and human disease

According to the minimum spanning tree generated from the comparison of the study isolates and 118 human isolates of *S. aureus* from Germany (Fig. 2), the results of the comparison between MLST types of study isolates displayed 17 groups randomly labelled A–S. Five of these groups (F, G, J, L and M) included isolates of human and bovine origin, indicating a similarity between isolates from these sources (Fig. 2). Several studies have identified the presence of host-specific genotypes of *S. aureus* (Smyth et al. 2009; Zadoks et al. 2002). The minimum spanning trees (Figs. 1 and 2) indicated the existence of several clonal complexes of closely related genotypes (lineages) within *S. aureus*. Our findings also showed the existence of a relationship between isolates from animals and from humans (Fig. 2), although separate clusters are common, as was shown by the presence of 12 clusters not containing any mixed origin strains. The majority of bovine mastitis milk-associated sequence types belonged to CCs which showed a close relation to sequence types of human origin (Fig. 2). This was especially seen for group F (CC 97), group G (CC 133) and group J (CC 151). These contained the majority of the bovine mastitis *S. aureus* in this study, and all of them clustered with strains of human origin. Recent studies demonstrated a close genetic relationship between MSSA isolated from milk and dairy products and the prominent human CC8, suggesting an exchange between human and bovine reservoirs (Resch et al. 2013). This is relevant to evaluate the risk of exposure for people involved in milking and consumers of raw dairy products alike.

Detection of enterotoxins encoding genes and staphylococcal virulence factors

Enterotoxin genes were identified in 37 (52.9%) of the isolates for more than one of the tested genes (Table 4), while 33 isolates (47.14%) did not possess any. In general, a relationship between genotype and toxin gene profile was found. Isolates belonging to CC151, CC79, CC479, CC5, CC8 and CC50 carried more toxin genes, while CC133 and CC398 contained none. The most frequently detected genes were *sei*, *sem*, *sen* and *selu*, which were found among the clonal complexes CC151, CC479, CC5 and CC50. *Seg* occurred in 26 isolates (37.1%; CC151, CC479 and CC5), while *seo* was found in nine isolates (12.9%; CC479, CC5, and CC50). S*ec*, *sed*, *selj* and *tst* genes occurred in eight isolates (11.4%), while *ser* was encountered in seven isolates (10%) and *seh* and *sep* in three (4.3%) of the isolates. All isolates were negative for

Fig. 1 Minimum spanning tree of the *S. aureus* isolates of this study ($n = 70$) combined with the results of the *S. aureus* isolates from different European countries ($n = 33$) selected from the MLST database http://saureus.mlst.net. The tree was constructed using BioNumerics version 7.5. The size of each circle indicates the number of isolates with the same sequence type. A thick solid line connects types that differ in only a single allele locus, and a thin solid line connects types that differ in at least two allele loci. The colours of the halo surrounding the MLST types indicate types belonging to the same group (A–G)


CC151
CC8
CC5
CC133
CC97
1380
479
504
151
2823
7
2825
133
2821
71
398
97
2824
2826
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2827
STs bovine mastitis in this study
STs human in Germany

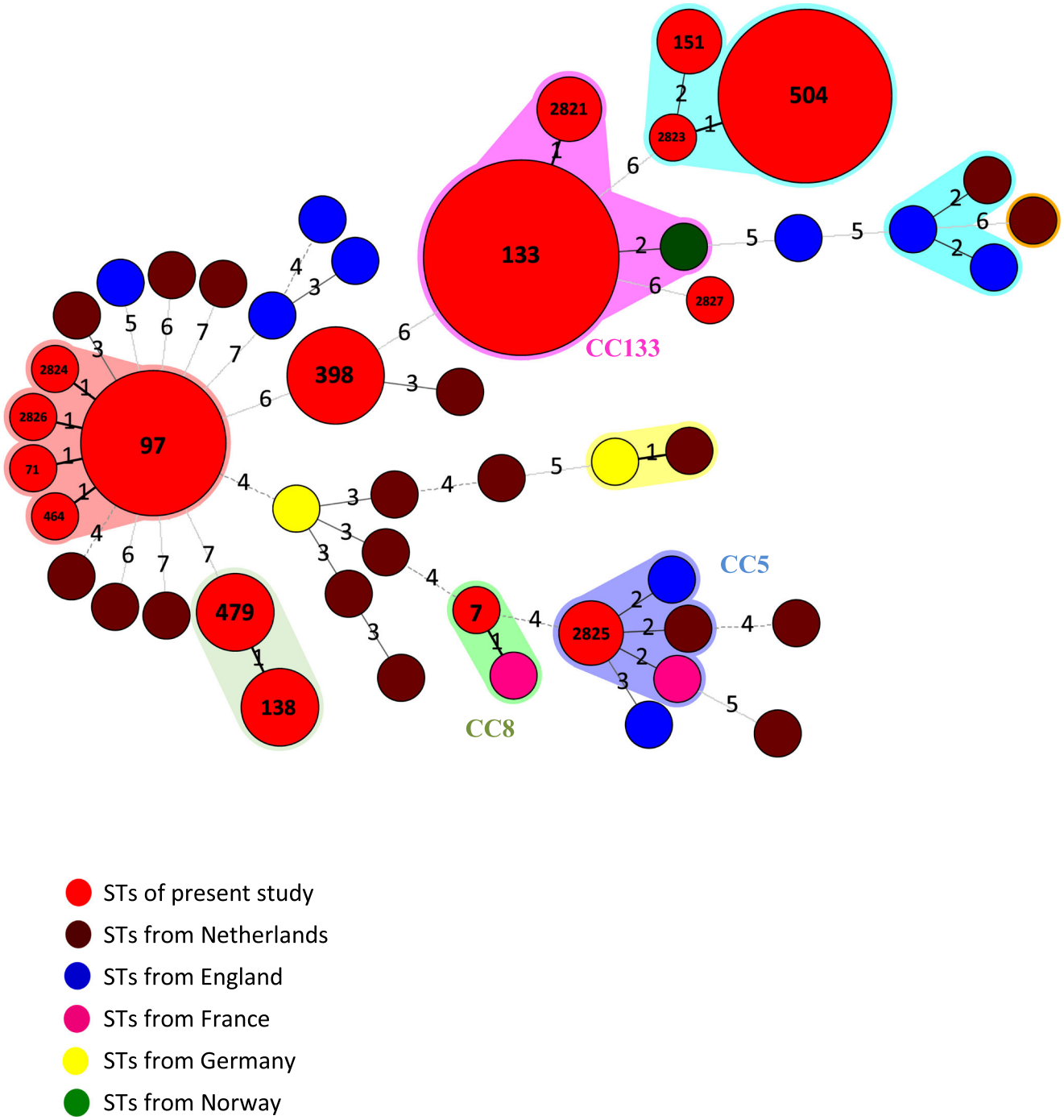

Fig. 2 Minimum spanning tree of the *S. aureus* bovine mastitis isolates of this study ($n = 70$) combined with human isolates from Germany ($n = 118$) selected from the MLST database http://saureus.mlst.net. The tree was constructed using BioNumerics version 7.5. The MLST types are displayed as circles. The size of each circle indicates the number of isolates with the same sequence type. A thick solid line connects types that differ in only a single allele locus, and a thin solid line connects types that differ in two allele loci. The colours of the halo surrounding the MLST types indicate types belonging to the same group (A–S)

sea, *seb*, *see* and *seq*. One possible explanation may have been the use of a single pair of primers which may not have recognised all allelic variants of these genes. Yet, the results confirm those of previous studies claiming that *seb*, *see* and *seq* could not be detected in staphylococcal isolates from bovine milk (Hummerjohann et al. 2014; Ote et al. 2011). Isolates with the clonal complex CC133 and CC398 were negative for all the tested enterotoxin genes in this study. Based on the presence of different enterotoxin genes, isolates were divided into nine different enterotoxigenic profiles (Table 4), suggesting a horizontal transfer of toxin genes among them. The most frequent enterotoxin gene profile I

Table 4 Profiles of genes encoding different staphylococcal enterotoxins, toxic shock syndrome toxin and staphylococcal proteins in the *S. aureus* isolates ($n = 70$) from bovine mastitis milk

Toxin gene profile	*Staphylococcus* enterotoxin	Isolates	
		n	%
I	*seg* + *sei* + *sem* + *sen* + *selu*	11	15.71
II	*sec* + *seg* + *sei* + *sem* + *sen* + *selu* + *tst*	7	10
III	*sed* + *selj* + *ser*	7	10
IV	*seg* + *sei* + *sem* + *sen* + *seo* + *selu*	6	8.57
V	*seg* + *seh* + *sei* + *sem* + *sen* + *seo* + *sep* + *selu*	2	2.85
VI	*sed* + *selj*	1	1.42
VII	*sec* + *sei* + *sem* + *sen* + *selu* + *tst*	1	1.42
VIII	*seh* + *sep*	1	1.42
IX	*sei* + *sem* + *sen* + *seo* + *selu*	1	1.42
0	No enterotoxin gene	33	47.14

(11 isolates, 15.7%) contained five different genes. Profiles II and III were detected in 10% of isolates, respectively. All other profiles were below 10%, profiles V to IX even being below 3%. Some enterotoxin genes are known to be grouped either as a gene cluster or organised as an operon (Terman et al. 2013). All isolates possessed the genes *coa*, *clfB* and *nuc*, whereas *clfA* was found in 69 (98.6%) of the *S. aureus* isolates.

In the present study, the most frequently occurring genes in *S. aureus* isolates were *seg*, *sei*, *sem*, *sen* and *selu.* These results were basically in agreement with those of Srinivasan et al. (2006). There are reports that, for instance, SEH caused (staphylococcal) food poisoning outbreaks in humans (Ikeda et al. 2005; Jorgensen et al. 2005). However, often several enterotoxins are present in a particular strain, and other toxin types like SEG, SEI and SElJ contribute to *S. aureus* food poisoning outbreaks as seen in molecular studies (Fisher et al. 2018). Especially, SEG, SEH, SEI, SEK, SEM and SEQ might play an important role in staphylococcal food poisoning. Nonetheless, immunological detection assays of the toxin have just recently become available and their epidemiological impact needs to be monitored (Fisher et al. 2018). The various types of enterotoxin genes of *S. aureus* isolated in this study and other studies can be attributed to the difference in the geographical region (Klein et al. 2012), the variation in utilised primers, types of samples, the source of samples and environments (Wang et al. 2012). In this regard, there is a need for improving and combining molecular and immunological assays in the future in order to be able to evaluate the presence of toxin genes and the prevalence of the toxin itself (Fisher et al. 2018).

Limitations of the study

The study was conducted on a set of isolates that were collected during routine diagnostics. Thus, these are not representative of the whole population of *S. aureus* involved in bovine mastitis cases. The comparison of the study isolates with database isolates included the data available at the time of the study and cannot be considered as a complete presentation of all available data, but should bring the results into context when compared the database strains. The present study only used one primer pair per enterotoxin type. More primer pairs would have possibly covered more allelic variants. However, the authors' focus was on investigating a broad spectrum of enterotoxins.

Conclusion

The genotypic characterisation of *S. aureus* isolated from bovine mastitis milk by MLST and *spa* typing showed that the infections in cow herds of different regions in Northern Germany were caused by different clones of *S. aureus*. Such types were also reported in association with bovine mastitis in different regions of the world. Thus, the types of *S. aureus* associated with bovine mastitis in Northern Germany do not seem to be unique to this region. Nonetheless, some novel STs and one novel *spa* type were identified typing of isolates. Furthermore, the comparison among study and database isolates indicates the existence of close relationships among isolates from animals and humans. Therefore, the importance to continue elucidating the relation between mastitis-associated *S. aureus* strains and those causing human diseases, particularly in terms of consuming contaminated dairy products, needs to be stressed in the future. This would be important in the context of the one health aspect.

Acknowledgements This manuscript is part of the thesis of Omar Sheet (2016), and additional data can be found there.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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